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Slow Genetic Divergence of *Helicobacter pylori* Strains during Long-Term Colonization

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The genetic variability of *Helicobacter pylori* is known to be high compared to that of many other bacterial species. *H. pylori* is adapted to the human stomach, where it persists for decades, and adaptation to each host results in every individual harboring a distinctive bacterial population. Although clonal variants may exist within such a population, all isolates are generally genetically related and thus derived from a common ancestor. We sought to determine the rate of genetic change of *H. pylori* over 9 years in two asymptomatic adult patients. Arbitrary primed PCR confirmed the relatedness of individual subclones within a patient. Furthermore, sequencing of 10 loci (~6,000 bp) in three subclones per time and patient revealed only two base pair changes among the subclones from patient I. All sequences were identical among the patient II subclones. However, PCR amplification of the highly divergent gene *amiA* revealed great variation in the size of the gene between the subclones within each patient. Thus, both patients harbored a single strain with clonal variants at both times. We also studied genetic changes in culture- and mouse-passaged strains, and under both conditions no genetic divergence was found. These results suggest that previous estimates of the rate of genetic change in *H. pylori* within an individual might be overestimates.

It is evident that *Helicobacter pylori* is highly adapted to the human stomach. It infects about one-half of the world's population, and no other natural reservoir is known. The infection is usually acquired before a person is 10 years old, and once established, it persists for life, unless it is treated with antibiotics (20, 31). When infecting a new host, the bacterium causes inflammation in the gastric lining, which in most cases is asymptomatic but can progress to a symptomatic chronic gastritis, gastric or duodenal ulcers, or gastric cancer (34). Bacterial, host, and environmental factors all contribute to the development of these *H. pylori*-associated diseases (10).

H. pylori preferentially colonizes the antrum of the stomach, where no acid-producing parietal cells are present, which results in a less acidic environment than that in the corpus. The growth is suggested to be patchy; i.e., *H. pylori* is present in isolated populations that differentially evolve at various locations, most likely in response to local selection pressures (9, 15, 22, 23). Early adaptation to the local microenvironment occurs through, e.g., altered expression of adhesins and lipopolysaccharide structures on the outer membrane through phase variation or intrachromosomal rearrangements, resulting in allelic variation (5, 18, 24, 29, 39, 44).

H. pylori is considered to be an exceptionally diverse bacterial species, and almost every individual harbors a distinctive bacterial population with clonal variants, as shown by the high interpatient genetic diversity of *H. pylori* strains (28, 33, 36). In contrast to many other bacterial pathogens, *H. pylori* does not show clonal spread in different human populations or within patient groups (37, 41). An exception is transmission within families, where clonality is observed and the same strain is most commonly found in mothers and children (21, 27). In addition, multiple-strain infections have been presented, but the frequency at which this occurs is uncertain (14, 16, 32, 42).

The relatively small *H. pylori* genome, which is approximately 1.65 Mb long, has been sequenced for two strains, strains 26695 (43) and J99 (4), and a comparison of these two genomes has shown that 6 to 7% of the genetic material differs (4). Most of the base pair differences are synonymous and do not affect the protein sequences. The natural competence of *H. pylori* and unusually high recombination and mutation rates may explain this strain variety (12, 25, 40). Furthermore, intrastrain recombination of identical repeats or between different alleles in the genome was recently suggested to contribute to the high intrahost strain diversity (7, 35, 38).

We have previously shown that *H. pylori* subclones differ both in genotype and in phenotype even though they are isolated from the same biopsy (11). In addition, Israel et al. used microarray genotyping to show that all subclones in one individual are unique, both within biopsies and between biopsies from different locations of the stomach (26). The aim of this study was to examine the genetic variability in *H. pylori* isolates from two adults at two times 9 years apart. In addition, we attempted to determine the rates of genetic change of 12 independent lineages of strain 26695 during repeated culture

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passages and of one clinical isolate after a 10-month infection of an Le^b-expressing transgenic mouse.

MATERIALS AND METHODS

Patients, strains, and culture conditions. Patients I and II were selected from a study of Gustavsson et al., which included 47 patients from Örebro County, Sweden (Gustavsson et al., submitted for publication). Patient I was 43 years old and suffered from epigastralgia when entering the study. Patient II was 29 years old and was seeking medical advice for gastric reflux problems. The *H. pylori* infections of these patients were not treated due to the standard treatment procedures in Sweden at the time of the first visit, when only patients with peptic ulcer diseases were treated for *H. pylori* infection.

Both patients underwent gastroscopy in 1990 and 1999. At the first endoscopy one biopsy from the gastric antrum was obtained, and at the second follow-up endoscopy two biopsies were collected, one from the antrum and one from the corpus region. The biopsies are referred to below as biopsies 90A, 99A, and 99C, respectively. The biopsies were stored at −70°C until they were cultured on GC agar plates as previously described (11). Single *H. pylori* colonies and a mixture of the total bacterial population were isolated from each biopsy for further investigation. From each patient, six subclones (subclones *a*, *b*, and *c* from the 90A biopsy, subclones *d* and *e* from the 99A biopsy, and subclone *f* from the 99C biopsy) were randomly selected for sequencing.

To study genetic changes in culture, we propagated 12 independent lineages of strain 26695 (43) by serial passage of single colonies (one cell bottleneck) on GC agar plates 100 times, which corresponded to approximately 2,500 generations of growth. The colonies were subcultured every 3 days. We previously colonized conventionally raised transgenic FVB/N mice expressing the human histo-blood group antigen $Lewis^b$ (the receptor for the BabA adhesin) on their gastric epithelial cells with *H. pylori* strain 67:21 for 10 months (11). Ten single-colony reisolates, designated 3:9:1 to 3:9:10, were isolated from one mouse and used in this study.

Genetic analyses. *H. pylori* DNA was prepared after 48 to 72 h of growth on GC agar plates using a QIAGEN DNeasy kit (QIAGEN, Hilden, Germany). PCR was performed using DyNAzyme DNA polymerase (Finnzyme, Espoo, Finland) or AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) according to standard protocols or as previously described (1, 2, 13, 41). Primers used in this study are shown in Table 1. Ten single-colony isolates from each biopsy were analyzed in two reactions using two arbitrary primed PCR (AP-PCR) primers, primers 1290 and 1283 for patient II and primers 1290 and D8635 for patient I (3). Primer D8635 was used for patient I due to an insufficient banding pattern with primer 1283. The 12 lineages of 100 in vitro passages of strain 26695 and the 10 reisolated isolates of strain 67:21 from a mouse were analyzed with primers 1290 and 1283.

Ten genetic loci, HP0887 (*vacA*), HP0142 (*mutY*), HP0071 (*ureI*), HP0115 (*flaB*), HP0954 (*rdxA*), HP0790 (*hsdS5*), HP0621 (*mutS*), HP0521, HP0547 (*cagA*), and the intergenic region between HP0547 and HP0549, were selected for sequencing to determine the base pair substitution rates. The gene nomenclature used in this paper is that of strain 26695 (43). DNA cycle sequencing was performed directly with PCR products using a BigDye terminator kit, version 3.1 (Applied Biosystems), and an ABI 3100 instrument. Size variations of four repetitive regions (*amiA*, *cagA*, and two regions of *cagY*) were investigated using PCR, as previously described (6, 7).

Mutation frequency analysis and antibiotic susceptibility tests. The frequency of spontaneous mutations was tested by using one subclone from each time and patient (subclones *a* and *d*) with a fluctuation test using selectivity for rifampin resistance mutations, as previously described (12).

To investigate naturally occurring resistance mutations, sensitivity to rifampin, amoxicillin, metronidazole, tetracycline, and clarithromycin was analyzed with subclones *a* to *f* from both patients, using E-tests (AB Biodisk, Solna, Sweden) on GC agar plates according to standard procedures.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under accession numbers AY571149 to AY571274.

RESULTS

AP-PCR revealed close intrapatient strain relatedness. We investigated the *H. pylori* population in two adult patients at two times separated by a 9-year interval. Biopsies were obtained from the antrum in 1990 (biopsy 90A) and 1999 (biopsy

TABLE 1. Primers used in this study

TIGR gene no. or use[a]	Forward primer (5′–3′)	Reverse primer (5′–3′)	Reference
HP0887 (*vacA*)	DLHPVacA-4 (ACA ACC GTG ATC ATT CCA GC)	DLHPVacA-3 (ATA CGC TCC ACG TAT TGC)	1
HP0142 (*mutY*)	mutY101(+) (AGC GAA GTG ATG AGC CAA CAA AC)	mutY102(−) (AAA GGG CAA ATC GCA CAT TTG GG)	1
HP0071 (*ureI*)	Hp71as(−) (GGC AAT GCT AGG ACT TGT)	Hp71S3(+) (TCC CTT AGA TTG CCA ACT AAA CGC)	1
HP0115 (*flaB*)	flaB:F (AAG GCA TGC TCG CTA GCG)	flaB:R (TAA TGT CTC TAG CGT CGG)	1
HP0790 (*hsdS5*)	726/790 SRU2 (TTT TGG ACG CTT TCA CAG AA)	726/790 Hp1SF2 (GCT GGG ATA CCG GCT AAT AA)	(A. Skoglund, personal communication)
HP0621 (*mutS*)	mutS3 (GGC AAA AA ACA AAC CCC TAT G)	mutS486 (CAA ACT TTC ATT CAA AGC)	12
HP0521 (putative)	CIF301 (CTT AGT GAA ATT AGT TGA AAA ATT G)	R11 (TGA GTT TTT GTG TCA TCA ACA G)	13
HP0547 (*cagA*)	CagAV F (AAG GGA ATT GTC TGA TAA)	CagAV R (TGA CAA ACT AGC AGG CAC TT)	This study
Region between HP0547 and HP0549	F69 (AAT GTT AAT ACA AAA GGT GGT TTC)	C2R130 (GCG GCG ATG TGG TTT GG)	13
HP0954 (*rdxA*)	rdx1 (GCC ACT CCT TGA ACT TTA ATT TAG G)	rdx4 (CGT TAG GGA TTT TAT TGT ATG CTA C)	30
HP0527 (*cagY*), middle region	MRRF (GCT TAC TGA ACC AAC AAA AAG TTC A)	MRRR (CGC TCA AAC CAT CCA AAC ACT TC)	6
HP0527 (*cagY*), 5′ region	FRRF (ATG AAT GAA GAA AAC GAT AAA TTG)	FRRR (CAC TTG AAC TTT TTG TTG GTT CAG)	6
HP0772 (*amiA*)	amiAF (GCG AGTGGT TAT CGG CTA GCT CC)	amiAR (CTT TCC CCC CAT GCC CAG CGT C)	8
HP0547 (*cagA*), repeat region	DUF130 (GGT GTA GGG CAA GCA ACG G)	R66 (TAG AAT CTT TGA GCT TGT CTA TC)	13
AP-PCR	1290 (GTGGATGCGA)		2
AP-PCR	1283 (GCGATCCCCA)		2
AP-PCR	D8635 (GAG CGG CCA AAG) GGA GCA GAC		2

[a] TIGR, The Institute for Genome Research.

TABLE 2. Overview of the nucleotide divergence (number of base pairs) in the 10 genes sequenced in subclones *a* to *f* from each patient

Gene	Patient I			Patient II		
	Length (nucleotides)	Nucleotide divergence in subclones *a* to *f*	Nucleotide divergence compared with 26695	Length (nucleotides)	Nucleotide divergence in subclones *a* to *f*	Nucleotide divergence compared with 26695
ureI	510	0	11	554	0	16
flaB	449	0	25	373	0	21
vacA	553	0	25	609	0	16
mutY	414	0	32	401	0	19
mutS	475	0	34	731	0	51
hsdS5	631	1 (subclone *d*)	—[a]	574	0	17
HP0521	570	0	31	955	0	—[a]
cagA	940	0	50	518	0	22
rdxA	623	1 (subclones *d*, *e*, and *f*)	25 (26)	624	0	20
HP0547-HP0549	412	0	—[a]	803	0	—[a]
Total	5,577			6,142		

[a] No similarity to strain 26695.

99A) and from the corpus in 1999 (biopsy 99C). Neither of the patients suffered from *H. pylori*-associated diseases. Ten independent colonies from most biopsies from the two patients were cultured; the only exception was the 99A biopsy from patient I, from which only seven colonies could be recovered. Two AP-PCRs were used to examine the relatedness of the subclones in each patient. The banding patterns of isolates from each patient were highly similar, and thus the intrapatient subclones originated from the same ancestral strain. However, as expected, the two patients carried unrelated *H. pylori* populations (data not shown). Twelve independent lineages of strain 26695 that had been subcultured in vitro 100 times were also analyzed by AP-PCR. The banding patterns of the lineages and the original wild-type strain were indistinguishable, which indicates that no major changes had occurred after 100 passages, corresponding to an estimated 2,500 generations of in vitro growth. In addition, no differences in the AP-PCR banding patterns were identified in the 10 clones reisolated from a mouse after 10 months of infection with strain 67:21.

Few nucleotide changes after 9 years of infection. Sequencing analyses were performed with three randomly selected subclones from each time and patient. Subclones *a*, *b*, and *c* were selected from biopsy 90A, subclones *d* and *e* were selected from biopsy 99A, and subclone *f* was selected from the 99C biopsy. Ten loci, *vacA*, *mutY*, *ureI*, *flaB*, *rdxA*, *hsdS5*, *mutS*, HP0521, *cagA*, and the intergenic region between HP0547 and HP0549, were sequenced in order to determine the base pair substitution rate in these loci during 9 years. Surprisingly, complete sequence concordance was found for these loci of all six subclones from patient II. For patient I, only a single base pair substitution was found in each of two genes (*rdxA* and *hsdS5*) (Table 2). Because of this low variability, the substitution rate could not be calculated. Furthermore, when we sequenced *ureI*, *vacA*, *rdxA*, *hsdS5*, and the intergenic region between HP0547 and HP0549 in 12 different lineages of strain 26695 that were in vitro passaged 100 times, the sequences were all identical.

Divergence in the *amiA* repetitive region. Four repetitive regions, regions of the *cagY* (5′region and middle region), *cagA*, and *amiA* genes, were selected for size screening by PCR. CagY is the major pilus-forming protein with antigenic variation properties (6), and CagA is an immunodominant effector protein of the type IV secretion system encoded by the *cag* pathogenicity island. The *cagA* repetitive region was stable, with one copy of the repeat in all isolates included in this study. In the two *cagY* regions, one subclone from biopsy 99A from patient II produced a band in the 5′ region that was approximately 400 bp larger than the bands produced by the other subclones from this patient. No other changes in size of the *cagY* fragments were detected.

The *amiA* gene, encoding an *N*-acetylmuramyl-L-alanine amidase homologue involved in bacterial cell wall synthesis, was recently shown to vary extensively in terms of the number of 15-bp direct repeats in isolates from one patient (7). We found pronounced differences in the two patients. In patient I, the PCR fragments were two sizes that were approximately 500 bp. While the colonies from biopsy 90A all yielded the larger fragment, the colonies from biopsy 99A all harbored the smaller fragment and the majority of the biopsy 99C colonies harbored the smaller fragment; however, 7% (2 of 30) of the biopsy 99C colonies contained the larger fragment. In patient II, PCR fragments that were four sizes, ranging from 600 to 800 bp, were amplified from different subclones from each biopsy. No differences were found in the in vitro or mouse-passaged strains.

Low mutation frequency. An unusually high frequency of *H. pylori* mutator strains has been reported, and about 30% of the clinical isolates have a mutation frequency as high as that of *Enterobacteriaceae* mismatch repair-deficient mutants (12). The mutation frequencies of two subclones from each patient, subclone *a* from the 90A biopsy and subclone *d* from the 99A biopsy, were measured using the frequency of rifampin resistance as the mutation indicator (12). The mutation frequency was approximately 10^{-8} in all subclones analyzed, and if this frequency reflects the mutation frequency in vivo, the number of substitutions is expected to be low in the *H. pylori* subclones from these patients (data not shown).

Isolates from the early time in patient I were metronidazole resistant. Antibiotic susceptibility tests were performed with three subclones from each time from each patient (subclones *a*, *b*, and *c* from biopsy 90A, subclones *d* and *e* from biopsy 99A, and subclone *f* from biopsy 99C), using five antibiotics. Most subclones were susceptible to rifampin, amoxicillin, tetracycline, metronidazole, and clarithromycin; the exceptions were

the subclones from the early time (subclones *a* to *c* from biopsy 90A) from patient I, which were resistant to metronidazole. Resistance to metronidazole is generally caused by changes of the intracellular redox potential within the cell. Sequencing of the *rdxA* gene, encoding an oxygen-insensitive NAD(P)H nitroreductase, revealed a previously described probable resistance mutation at position 200 (changing the amino acid from alanine to valine) (38) in subclones *a* to *c* from the 90A biopsy from patient I (Table 2). Additional analyses of subclones from patient I (10 subclones from biopsy 90A, seven subclones from biopsy 99A, and 10 subclones from biopsy 99C) revealed that all of the biopsy 90A subclones were resistant and all of the biopsy 99A and 99C subclones were susceptible to metronidazole, indicating the presence of a time-specific antibiotic-resistant clone.

DISCUSSION

We characterized several randomly chosen subclones of the *H. pylori* population in two patients over a period of 9 years. Each patient was colonized with one distinct population, as determined by AP-PCR. When we sequenced 10 loci (~6,000 bp) in three subclones from each time and patient, only two base pair differences were found in one patient, while there were no changes in the other patient. However, further analyses by PCR of the repetitive region in the *amiA* gene revealed genetic variation among the subclones. The few nucleotide differences found by sequencing made it impossible to calculate the rate of change between the two times. However, such calculations have been described previously (17). In the study of Falush et al. (17), a single colony was isolated from each of two biopsies from 26 individuals with a mean of 1.8 years between the two endoscopies, and 10 genes were sequenced for each isolate. From the nucleotide differences found between the times, the median DNA import size was calculated to be 417 bp and the recombination frequency was calculated to be 60 imports, resulting in replacement of 25,000 bp per genome per year. If the recombination rate estimate is correct, one recombination event that increased fitness and allowed a selective sweep would take place each week. The estimates of these workers may be overestimates, since they did not take into account the genetic variability that may exist in the *H. pylori* population at a certain time and thus the observed differences might have been present at the time of the first endoscopy. On the other hand, the individuals in the study of Falush et al. originated from two high-prevalence areas, Louisiana and Narino in the Andes of Colombia, whereas the two patients in this study originated from a low-prevalence country, Sweden. The number of transiently infecting *H. pylori* strains may therefore differ in the two studies, explaining the discrepancy in the observed nucleotide divergence. Furthermore, isolates that were obtained from the two patients included in the present study exhibited relatively low mutation frequencies compared to other strains of *H. pylori* (12). It is possible that the high sequence similarity between the subclones analyzed reflects this low mutation frequency. The mutation frequencies of the isolates included in the study of Falush et al. are not known, but the presence of mutator strains in the bacterial populations would explain the observed higher degree of diversity.

It is clear that *H. pylori* strain diversity is extraordinary, but where and when is it generated? It is difficult to reconcile the comparably small number of genetic changes accumulated over 9 years in our two patients with the fact that every person harbors his or her own unique *H. pylori* population. To account for our findings for our two patients, we propose two possible scenarios for the generation of variability in *H. pylori* with either monoclonal or polyclonal transmission. In the first scenario, a single clone may initially diverge rapidly within the microniches of the stomach to adapt to the new environment, but as soon as the niches are occupied, the rate of change is reduced. The genetic changes may also occur in a stepwise manner, if there is high variation in selection pressures. According to this model, most of the clonal variability was generated during the childhood of the patients (assuming that they were infected early in life) and therefore was already present at the first time examined, when the patients were 43 (patient I) and 29 (patient II) years old. Then only minor changes occurred in the *H. pylori* populations of patients I and II over the 9-year study period due to low selective pressure and rather constant environmental conditions. Alternatively, transmission is polyclonal, and the transmitted clonal variants evolved as a divergent population in several generations of hosts. Consequently, the genetic variability observed in any specific host is the sum of all the genetic variation accumulated during all previous host passages. With this hypothesis, the time span of our analysis (9 years) is too short, and an examination of strains transmitted vertically from several generations of hosts is needed to test this model. To examine the suggested scenarios, we suggest three possible approaches. First, infants in areas with high *H. pylori* prevalence could be monitored over a prolonged period and sampled for *H. pylori* with short time intervals. This experiment is not ethically feasible as long as *H. pylori* can be cultured only from gastric biopsy material. Another approach would be to infect adult volunteers with one defined clone and monitor the genetic divergence in the population during the initial colonization phase and after a prolonged time (19). Finally, one could monitor the genetic variability among strains isolated from a mother and her offspring after vertical transmission (27).

In summary, we found far less variation and slower genetic divergence of *H. pylori* during long-term colonization than might have been expected based on previous reports. We propose two models for the generation of variability among *H. pylori* populations and how to approach these scenarios in future studies.

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